MINIREVIEW

Enzymatic approaches in paper industry for pulp refining and biofilm control

C.E. Torres, C. Negro, E. Fuente and A. Blanco

Chemical Engineering Department. Faculty of Chemistry.

Complutense University of Madrid. 28040. Spain

***Angeles Blanco**

Chemical Engineering Department

Faculty of Chemistry. Complutense University of Madrid

Av. Complutense s/n. Madrid 28040, Spain

Tel, +34 91 3944247

Fax, +34 91 3944243

e-mail, ablanco@quim.ucm.es

ABSTRACT

The use of enzymes has a high potential in the pulp and paper industry to improve the economics of the paper production process and to achieve, at the same time, a reduced environmental impact. Specific enzymes contribute to reduce the amount of chemicals and energy required for the modification of fibers and helps to prevent the formation or development of biofilms. This review is aimed at presenting the latest progresses made in the application of enzymes as refining aids and biofilm control agents.

KEYWORDS: Enzymes, Refining, Biofilm, Paper industry.

INTRODUCTION

Although there is a wide variety of paper products and process layouts almost all of them have the following basic units (Figure 1):

- Pulping: consisting of a series of mechanical or/and chemical operations to obtain a suspension of cellulosic fibres (pulp) from lignocellulosic raw materials (wood, recovered paper or even annual plants).
- Stock preparation: removes detrimental material and air and conditions the pulp by developing the strength properties of the fibres by means of refining when necessary.
- A paper or board machine: where the sheet is formed by draining the water of the suspension through a wire then, pressing the wet sheet and, finally drying it by heating in the drying section.
- Finishing section: depending on the paper or board grade, there are additional process units like calenders, sizers, coaters, winders, rewinders, sheeting plant and a roll wrapping station.

Pulp and paper manufacture from wood is a chemical-intensive process (Figure 1). Solutions of NaOH, NaS_2, Na_2SO_3, or other chemicals are used for cooking wood chips to dissolve the lignin and hemmicelluloses and release the cellulose in the case of preparing chemical or semichemical pulps. NaOH, silicates and surfactants are used during pulping and deinking of recovered paper. Combinations of chlorine compounds and/or peroxides, ozone and other oxygen-based chemicals are used during bleaching. Furthermore, many other compounds and polymers are used as process aids or product aids during stock preparation and in the paper machine to improve the process and to meet the functional requirements of the different

types of paper; retention aids, strength agents, sizing aids and coating binders, are only some examples.

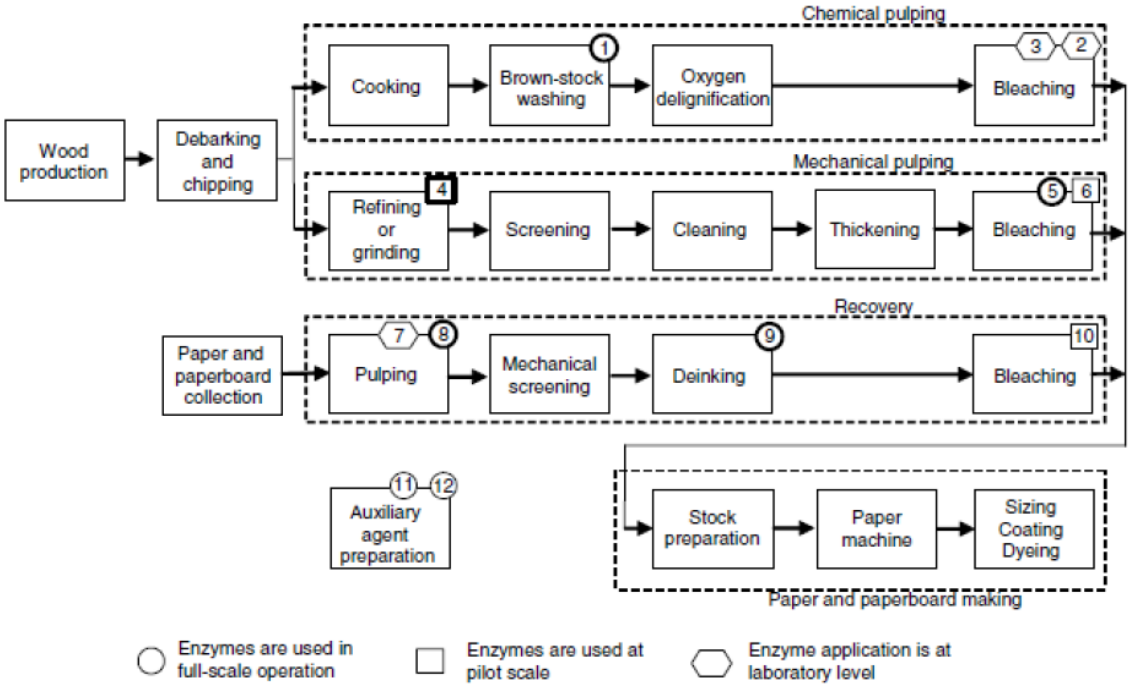

Figure 1. Main processes in the pulp and paper industry with indication of present and potential points of enzyme application (adopted from Skals et. al., 2008).

Over the past decades, biotechnology has gained ground in the manufacturing of a variety of industrial products, including pulp and paper, in an attempt to provide more friendly processes and to reduce costs. Research in this sector has focused essentially on 'green' catalysts or enzymatic products. They are often superior to their conventional alternatives in terms of raw material and energy consumption yield and/or of the quality of the final product, due to their higher specificity and efficiency (Skals 2008).

The main focus areas of enzyme technology in the paper industry are as follows (Figure 1): (i). biopulping to save energy (Hart 2009; Lecourt 2010) and replace harmful chemicals (Li 2010), (ii) water treatment, and (iii) to solve many problems (Poorna and Prema 2007) related to deinking (Bajpai 2010), drainaibility, hornification, deposits of pitch (Blanco 2009) and stickies, and biofilm formation (Torres 2011).

The present review covers the last years and it is focused on the enzymatic application for refining and biofilm control.

REFINING

Refining consists of mechanically separate and modify fibres by means of a device that applies compression and shear forces onto the wetted chips or fibres, to obtain fibers able to interact among them to form a network. The refining process presents two stages: (i) fibre separation and (ii) fiber development, which can occur simultaneously during the first stage. In the first stage, wood chips are broken into individual fibers by means of counter-rotating disks. Fiber development consists of causing fibrillation and increasing flexibility of the fibers to improve the fibre properties.

The overall objectives of refining are: (i) increase the ability of fibres to establish links between them to produce a sheet of paper with higher strength, and better impression properties and (ii) shorten too long fibres to improve formation (homogeneity of the sheet) and develop sheet properties such as the absorbance, porosity and opacity.

During refining, fibres are forced to pass between a rotor and a stator, in a PFI mill, or between counter-rotating disks, if a disk refiner is used. Raised areas of "bars" on each surface expose

the fibres to repeated compression and shearing forces. These forces gradually delaminate and peel the fibre cell wall. Primary (P) and outer secondary wall (S_1) are peeled off during refining, leaving the inner secondary wall (S_2) exposed (Figure 2).

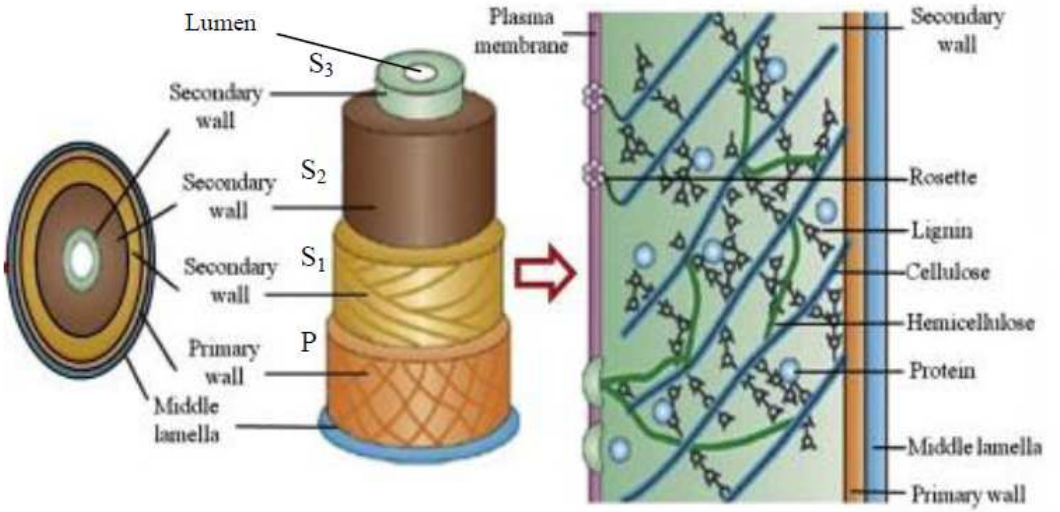

Figure 2. Wood fibre structure. P: primary cell wall, S2: middle secondary cell wall, S1: outer secondary wall, and S3: inner (tertiary) cell wall (adapted from Menon and Rao, 2012)

Therefore, refining causes the following effects: (i) external fibrillation, which is a peeling off of fibrils from the fiber surface, while leaving them attached to the fiber surface, these fibrils can be as thin as a chain of cellulose and increase notably the surface able to interact with other fibers, compounds and particles in the pulp suspension. (ii) Internal fibrillation or swelling, which is caused by the breakage of hydrogen bonds among microfibrills and increases the water absorption, the specific volume and the flexibility of the fiber (Maloney and Paulapuro 1999). (iii) Collapse of the lumen due to the compression of the fiber, and (iv) generation of fines due to the cutting action of the refiner elements (disks or rotor and stator). As a consequence of these effects, refining decreases drainage rate of the water from the pulp and increases the water retention in the pulp. Therefore, the quantification of the refining result is based on the measurement of the freeness and the water retention ability of the pulp. The Canadian Standard Freeness (CSF) is a measurement of the volume of excess water from overflow when the pulp is drained at normalised conditions described in the Tappi test method T 227 om-99. it is an indirect measurement of the drainage rate of the pulp and it is mainly affected by the external fibrillation and the amount of fines. The water retention value is the measurement of the amount of water that remains in the pulp after its drainage at normalised conditions according to ISO 23714:2007 and it is mainly affected by the internal fibrillation. The lower value of CSF and the higher value of WRV are related to a higher refining action.

The intensity of each effect depends on the type of refiner and the design of the refiner elements. For example, PFI mill causes a high fibrillation and lumen collapse of the fibres, but disk refiners have a higher cutting action. However, there are other many variables that affects to the refining action e.g. furnish composition, fiber morphology, kind of pulp, pulp consistency, refining energy and how this energy is transmitted to the fibers through the mechanical action. Specific refining energy (SRE) is the product of the impacts imposed per unit of mass of pulp, N, by the energy of each impact I, as shown in equation [1] (Kerekes 1990). Both N and I are function of a C-factor as indicated in equations [2] and [3]. C-factor represents the ability of the refiner to imposed impact upon pulp fibers and it is a function of the geometry of refiner elements, pulp consistency, rotation speed, and fiber coarseness and length.

$$SRE = I \times N \quad [1]$$

$$I = \text{Net power} / \text{C-factor} \quad [2]$$

$$N = \text{C-factor} / \text{Pulp mass flow} \quad [3]$$

When a discontinuous refiner is used, N is proportional to the number of revolutions made by

the refiner elements during the refining process at a constant pulp consistency and type.

Refining can be carried out at high consistency or at low consistency. The main difference is the larger gap among the refiner elements in the high consistency refiners, which, in addition, operate at higher speed. This allows feeding pulp with a consistency up to 35%. Low consistency refiners operate with pulp at consistency up to 6%.

The energy required by the refining stages can represent a considerably high part of the total energy consumption of the pulping process, especially in the case of chips refining because of the cementing action of lignin. Therefore, chips are pre-treated to degrade lignin, with steam, in the case of thermomechanical pulps (TMP), or with chemicals, in the case of chemical pulps.

However, cellulose, hemicellulose and lignin can be modified and degraded in nature by a number of microbes by means of a vast array of enzymes. Therefore, the application of enzymes to wood chips or to fibers is an attractive alternative way to decrease energy demand in the refining process and to introduce novel functional properties on fibers (Maloney and Paulapuro 1999).

The interest in the bio-modification of cellulose fibers, has grown progressively in recent years because it reduces energy consumption and the damage to the fibers caused by refining.

Enzymes involved in improving refining

Biopulping makes use of the enzymes generated by fungi to reduce the consumption of chemicals in the pulping stage of wood chips (Ferraz 2008; Singh and Chen 2008) to increase the yield of fiber, to reduce further refining energy requirements, or to provide specific fiber modifications (Kenealy and Jeffries 2003; Singh 2010). Understanding biopulping mechanisms is of high relevance since more resistant and competitive fungal species could be selected to increase the process efficiency. Some advantages and disadvantages are summarized in table 1. (Singh 2010).

Enzyme treatment of wood chips can open up the cell wall structure, and hence, lead to fiber separation at preferable locations in subsequent refining. When more fiber separation takes place in the secondary wall, more cellulose fibrils will be exposed on the fiber surface, which benefits inter-fiber bonding. Since wood chips are treated at high temperature and basic pH, the enzymatic procedures require proteins exhibiting a high thermostability and activity in a broad pH range (Haki and Rakshit 2003). Enzyme treatment of wood chips or coarse fibers in mechanical pulping may lead to significant energy saving in refining, but more research should be carried out to develop applicable technology at industrial scale (Li 2010). The enzymes with effects on refining are described below.

Cellulase

Cellulases are complex enzymes, whose enzymes act synergistically and are subdivided into at least three different activities. In this way, endo-ß-1,4-glucanases or endoglucanase act randomly breaking internal molecular bonds in the amorphous regions of the fibers, producing a rapid decrease in the length of the chain and a slow increase in the quantity of free reducing groups. The exo-ß-1,4-glucanases or cellobiohydrolases remove glucose or cellobiose units from the end free non-reducing cellulose chain, resulting in a rapid increase in sugars or groups reducers and little change in the size of the polymer. Finally, ß-1,4-glucosidase hydrolyzes the cellobiose produced by the above activities, giving glucose as end product. The endoglucanases and celobio-hydrolases act by reducing the molecular weight of cellulose, leading to a decrease in viscosity of the medium (Gandinia and Pasquini 2012). Cellulases act on the surface and inner layers of cellulose fibers in an efficient enough way to allow the production of special paper with reduced energy consumption. Cellulase effects on fiber morphology lead to improved fiber–fiber bonding in the refining process and hence to increased fiber cohesion in the final paper (Cadena 2010; Kaur 2010; Kuhad 2011; Luo 2011; Ponce and Perez 2002).

Xylanases

Xylan, which is the dominating component of hemicelluloses, is one of the most abundant organic substances on earth and its biodegradation is performed by xylanolytic complex (enzymes) produced by fungus and bacteria. Xylanases (endo-1,4-ß-xylanase) are glycoside hydrolases catalyzing the endolytical hydrolysis break down hemicellulose by hydrolyzing the

β(1,4)-linkages of xylan backbone structures without environmental pollution (Dhiman 2008, Savhita 2009, Wang 2010). Based on pore size distribution it has been demonstrated that xylanases can easily penetrate inside the cell wall and, therefore, alter the dewatering properties of the cell wall (Blomstedt 2010, Maloney and Paulapuro 1999, Ponce and Perez 2002, Senior 1988).

Laccase

Laccases (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) belong to the so-called blue-copper family of oxidases. They are glycoproteins, which are ubiquitous in nature. Laccase enzymes are expressed by white-rot fungi and other organism that play a crucial role in the terrestrial carbon cycle by helping to degrade lignocellulosic material. Lacases have been reported in plants and virtually in every fungus that has been examined (Rashmi and Nishi 2010). Laccases are polyphenol oxidases that are found in many plants, fungi, and microorganisms; they act on phenolic substrates by catalyzing the oxidation of their phenolic hydroxyl groups to phenoxy radicals while dioxygen is reduced to water; this initiates the depolymerization process. (Waung 2010). Laccases also oxidize lignin. Laccase is a large molecule (MW ~70,000) which cannot penetrate deep into wood; moreover, due to its rather low-redox potential (~0.5–0.8 V), it is unable to oxidize nonphenolic (C4-etherified) lignin units, which have a high-redox potential (>1.5 V). Because of these limitations, laccase alone can only oxidize phenolic lignin units (<20% of all lignin units in native wood) at the substrate surface. Therefore, laccase is often applied with an oxidation mediator, a small molecule as 2,2´-azinobis(3-ethylbenzthiazoline- 6-sulfonate) (ABTS) or 1-hydroxybenzotriazole (HBT) among others, able to extend the effect of laccase to nonphenolic lignin units and to overcome the accessibility problem. This association is named laccase mediator system (LSM). In these so-called LMS, the mediator is first oxidized by the laccase and then diffuses into the cell wall, oxidizing lignin inaccessible to laccase (Cadena 2010; Camarero 2007; Riva 2006: Widsten and Kandelbauer 2008).

Manganese Peroxidase

Lignin peroxidases (LiP) are generally considered the primary catalysts for the fungal cleavage of nonphenolic lignin structures. Manganese peroxidase (MnP), oxides Mn^{2+} to Mn^{3+}, which oxides phenolic structures to phenoxyl radicals. The product Mn^{3+} is highly reactive and complex with chelating organic acid, as oxalate or malate, which are produced by the fungus. The redox potential of the Mn peroxidase system is lower than that of lignin peroxidase and it has shown capacity for preferable oxidize in vitro phenolic substrates. On the other hand, studies indicate that contrary to LiP, MnP may oxidize Mn(II) without H_2O_2 with decomposition of acids, and concomitant production of peroxyl radicals that may affect lignin structure (Maciel 2010).

Amylase

Amylases hydrolyze starch. Starch contains about 15–30% amylose and 70– 85% amylopectin. Amylose is a long linear polymer of α-1,4-linked glucose residues. Amylopectin is a branched polymer having both α-1,4 and α-1,6 linkages. Three types of amylases are involved in starch bioconversion: endo-amylase (α-amylase), exo-amylases (glucoamylase or glucan 1,4-α-glucosidase, β-amylase), and debranching enzymes (pullulanase or isoamylase). α-Amylase cannot act on α-1,6 linkages and hydrolyzes internal α-1,4-glycosidic bonds of starch randomly and produces malto-oligosaccharides of varying chain lengths. Glucoamylase cleaves glucose units from the non reducing end of starch and it can hydrolyze both α-1,4 and α-1,6 linkages of starch. β-Amylase hydrolyzes the α-1,4-glycosidic bonds in starch from the nonreducing ends. Pullulanase (pullulan α-1,6-glucanohydrolase) or isoamylase (glycogen α-1,6-glucanohydrolase) cleaves the α-1,6- linked branch points of starch and produces linear amylosaccharides of varying lengths.

Pectinases

Pectin is a main component of the middle lamella and primary cell wall of the cellulose fibres. The term pectin is used for a group of components comprising rhamnogalacturonans, galactans and arabinans. Rhamnogalacturonan is a main component of pectin and has a backbone of α-(1,4)-linked D-galacturonic acid units and α-(1,2) or α-(1,4)-linked L-rhamnose. Pectinases are the group of enzymes involved in depolymerisation of the pectic polymers. This group of enzymes mainly consists of polygalacturonases (PG), pectin methyl esterases and pectin lyases

polygalacturonases cleave the bonds between galacturonic acids of the pectin chain. It is a hydrolytic enzyme and exists in two forms: endo-PG and exo-PG. Endo-PG acts randomly on the α-(1,4)-polygalacturonic backbone, whereas exo-PG acts at the non-reducing end of the chain. Pectin methyl esterase provides demethylation of pectin and decrease the amount of esterified pectin.

Enzymatic effects on virgin fibers

The main effect of enzymatic treatments is that wood chips become softened and more porous. Consequently, these treated chips are more easily broken apart during pulping and specifically during refining. This will reduce substantially the energy consumption during refining. The main results obtained are summarized in tables 2 and 3.

Mechanical Pulps

The biopulping process, where wood chips are treated with white-rot fungi prior to mechanical pulping, has been successfully applied to decrease the high demand of energy in refining and to improve pulp properties.

Akhtar et.al. (1999) carried out a study about the biomechanical pulping. The control and fungus-treated chips were refined through a thermomechanical pulp mill (TMP) producing lightweight coated paper. The fungal pretreatment saved up to 33% of electrical energy and improved the paper strength properties significantly compared to the control. These results are summarized in table 4.

Lou et al. (2011) studied the effects of cellulase modification on the surface and quality of masson pine fiber of mechanical pulp. The treatment with 75 IU/g (to bone dry materials) of enzyme, at 50ºC, during 150 min, at a pH of 5.5 and for a pulp concentration of 15%. produced a smooth modification of the fiber surface. The surface roughness was improved because the middle lamella, primary wall and outer layer of secondary wall were broken.

Maijala et al. (2008) tested 100 $U.g^{-1}$ of MnP produced by *Bjerkandera adusta* from JenaBiosGmbH(Germany), laccase produced by *Trametes versicolor*, and pectinase (*Aspergillus*) from Fluka (Germany). The consumption of energy for refining was examined with a laboratory low-intensity refiner after 6-h enzyme treatments. The work conditions were: 10% of consistency, pH 5 and 40ºC. The specific energy consumption in the refining of Scots pine wood chips treated with MnP decreased about 11%, and in the refining of Norway spruce somewhat less, 6%. Hydrolytic enzyme (pectinase) and MnP treatments on pine resulted in similar energy savings on average, though the hydrolytic enzyme treatments at their best reached about 16% energy savings. They induced: (i) changes in the wood structure which do not correlate to the total energy consumption and (ii) an increase of the surface charge of the fibers in the case of MnP treated pine pulps refined to reach a value of CSF between 85 and 130 mL was absorbed. Most laboratory handsheet properties, i.e. strength, light scattering and opacity, were improved at a given specific refining energy. Only brightness was slightly decreased.

Studies performed on wood chips treated with xylanase (0-50 AXU/g) prior to a CTMP process (consistency 10%), shows that after xylanase treatment of wood chips at 40-65ºC, during 240 min, fiber separation occurs more often between the S_1 and S_2 layers (Fig. 2). In contrast, without xylanase treatment, the majority of separations occur in the middle lamella region. Xylanase hydrolyzes only xylan in wood. When xylan in the secondary wall is removed or degraded, the fiber wall structure will open up at the particular points, and in the subsequent refining process fiber separation will take place more likely along the weakened points. Furthermore, if fiber separation takes place in the secondary wall, less energy would be required for fiber development in the second stage of refining (Li 2010).

Eucalyptus grandis wood chips biotreated during 60 days by *Ceriporiopsis subvermispora* in a 50-tonne chip pile have been evaluated for TMP and CTMP processing at mill scale. *Ceriporiopsis subvermispora* is a white-rot basidiomycete, its principal activity is related with MnP-initiated lipid peroxidation reactions for degradation of non-phenolic lignin substructures (Savhita 2009).

The process presented a 18% reduction of the average energy consumption (from 913 to 745 kWh/tonne) for producing TMP pulps with CSF 450–470 mL . In the case of CTMP pulps with similar CSF, energy saving consumption was 27% (1038 to 756 kWh/tonne). Bio-treated samples are softer and refine well even in lab-scale refiners, giving good quality fiber. However,

since some fiber damage occurred during lab-scale disk refining of untreated wood chips and this damage did not occurred at mill scale, the improvement in mechanical properties of the pulps prepared at industrial scale contrasted with significant improvements observed in lab-scale pulping experiments. Tensile indexes data from several *Eucalyptus grandis* pulps prepared at lab scale indicated that these pulps were very weak as compared with mill pulps. (Ferraz 2008).

The treatment with Novozym 476 (Cellulase) shows a considerable saving of electricity in the second stage of refining and in the reject refiner by softening cellulose fibers (-160 kWh/t pulp). Combustion of wood chips does not add to fossil energy consumption and does not contribute to global warming, and this explains why the advantages of enzyme application are greater for these two impact categories than for acidification, nutrient enrichment and photochemical smog formation (Skals 2008).

In the study performed by Kazymov (2010) the effects of pectinase, endoglucanase and a mixture of enzymes on three different size raw materials were tested: normal size chip, crushed chip and water impregnated, instantly preheated, pressed and then fiberized at 400 kWh/t chip further named fiberized pulp. 5 kg/t of endoglucanase reduced the energy consumption by 20% while the use of 1.5 kg/t of the mixture of enzymes produced a decrease of about 15% of energy consumption during refining. Pectinase at different dosages to a maximum of 5 kg/t and different treatment times did not show significant effect on energy consumption. These results differ from those obtained by Sabourin and Hart (2010) they applied two pectinase treatments to TMP of black spruce (*Picea mariana*) wood chips and allowed to react for a period of 2.5 h. The average temperature during the reaction period was 47-48 ℃. Enzymatic effects were studied on two refined pulps (1800 PFI revolutions). Pectinex 3XL® is a polygalacturonase, the enzyme protein used was separated and purified from *Aspergillus aculeatus* and *Aspergillus niger*. The application dosage was 720 g/t wood. The Novozyme 863® was a more aggressive enzyme preparation produced by a selected strain of *Aspergillus aculeatus*. This enzyme preparation contains polygalacturonase, other pectolytic activities, and a range of hemicellulolytic activities. It has the ability to disintegrate wood fiber cell wall material and works well in the temperature range of 25-50℃. The application dosage was 830 g/ t wood. The specific energy consumption was reduced by 9% and 9.6% respectively. The Pectinex 3XL® enzyme treatment successfully enhanced the tensile and tears indexes of the resulting pulp through specific surface activity in a desirable way while Novozyme 863® was somewhat detrimental toward some of the desired pulp properties (Sabourin and Hart 2010).

Chemical Pulps

Yang (2010) studied the effects of cellulose and xylanase treatment (20-30 IU/g) on PFI refining of mixed poplar pre-conditioning refiner chemical alkaline peroxide mechanical (P-RC APMP) pulp. Operational conditions were by cellulose at 55ºC, and pH 6 and by xylanase at 40ºC, and pH 6.5, both treatments were applied during 90 min. The results showed that the refining of pulp treated by enzyme was improved, e.g. an increase in refining degree (CSF decreased from 1121 to 946 mL) and a decrease of 10%-25% in energy consumption at the same refining degree. Breaking length, tearing index, bursting index and folding endurance of the pulp treated by cellulase were respectively improved by 18%, 14%, 16% and 100% at the same PFI revolutions. Brightness of the pulp treated by xylanase was increased by 1.7 percent ISO, and physical strengths of the treated pulp were slightly improved. Effect of cellulase-treatment is better than that of xylanase-treatment in improvement of refining, decreasing energy consumption and intensification of physical strength. In similar studies performed by Yang (2011) it was observed that the effects of cellulose pre-treatment on the freeness and energy consumption of alkaline peroxide mechanical pulp of *Populus tomentosa* (fast-growing poplar) showed a decrease of freeness in the range of 30 mL to 55 mL at the same number of revolutions, and a decrease of PFI mill revolutions from 1000 to 15000 rpm when reaching the same freeness, compared with the unpreatreated pulp. This is related to a decrease of energy consumption in the range of 12% to 22%. On the other hand, the effects of xylanase pre-treatment on the same pulp, compared with the unpretreated sample, showed a decrease of freeness in the range of 25 mL to 50 mL, at the same revolutions, and a decrease of PFI mill revolutions in the range of 1000 to 4500, when reaching the same freeness, which means a decrease of refining energy consumption in the range of 12% to 18%.

Hart in 2009, impregnated *Eucalyptus* wood chips with various blends of fiber modifying enzymes prior to preconditioning refiner chemical-alkaline peroxide mechanical pulp

processing. A total of four different enzyme blends were employed in this work. Enzymes were applied at 40-51ºC during 46 min. These enzymes were obtained from *Trichoderma reesei* and contained various xylanase and/or cellulose components. The process includes chemical pre-treatment and two stages of refining. The energy consumption was compared at the same CSF level of 350 mL. Some enzyme treatments were found to reduce SRE by at least 24%. The enzyme hydrolysis within the cell wall was observed by transmission electron microscopy of impregnated chips with high spatial resolution. The enzyme blends that successfully reduced SRE requirements were found to selectively loosen the bonds between the S1 and S2 layers of the fiber wall. Enzymes which selectively attached the S2 layer did not impart any SRE reduction (Hart 2009).

Ko studied in 2011 the treatment with FiberCare® of the unbleached kraft pulps from *Eucalyptus globulus*. The enzymatic treatment was applied during 24 h at pH 6 and 40ºC. The result obtained after refining by 10000 revolutions with PFI the treated sample was 331.7 ± 5.8 mL while for the untreated sample was 314.0 ± 5.0 mL CSF. Fibrillation by PFI refining clearly increased the fiber widths, which was responsible for more available surface area. In addition, the increased fine content provided additional binding sites for the enzyme, although PFI refining reduced fiber length.

A synergistic action of enzymes from the same bacterial isolate *Bacillus pumilus* was evaluated for the prebleaching of a kraft pulp (a mixture of 82–84% mix hardwood and 16–18% bamboo pulp). *Bacillus pumilus* exhibits good cellulase-free xylanase and pectinase production (in the ratio of 5:1). The optimal conditions for the enzymatic treatments were: temperature of 55ºC, retention time of 180 min, pH of 8.5 and the xylanase–pectinase dose of 4.5 and 0.9 U/g. The enzymatic prebleaching of kraft pulp resulted in 8.5% reduction in kappa number of the pulp, showing remarkable delignification by the enzyme treatment. This approach resulted in a 25% reduction of active chlorine consumption in subsequent bleaching stages without any decrease in brightness. Furthermore, a significant increase of pulp quality was achieved: increase in burst factor (9%), tear factor (4.6%), breaking length (4.4%), double fold number (12.5%), Gurley porosity (4%) and viscosity (11.8%). These results indicated that enzymatic prebleaching facilitated an increase in pulp fibrillation, water retention and restoration of bonding in fibers (Kaur 2010).

In another case, Lecourt 2011 tested a LSM system (Laccase + mediator) on a *Pine radiata* unbleached kraft. The system was effective at 40ºC, pH 7, 4.5% consistency and treatment time of 30 min. The laccase, alone or associated to the new mediator, facilitated the refining and breaking length was enhanced. The fibers were more amenable to the mechanical action and fiber fibrillation and cutting requested less energy. This was confirmed by the higher macrofibrillation index observed on the refined biotreated pulps (Lecourt 2010).

Several laboratory experiments have been performed in which bleached kraft pulps (BKP) were subjected to different enzymatic treatments. Michalopolos (2005) applied FiberZyme LBR®, a formulation that contains the *Chrysosporium lucknowense* cellulase noted as CellA. CellA (120-160 mL/t), was applied at 40ºC, pH 7.0, during 300 min, on pulps both hardwood (*Eucalyptus globulus*) and softwood (Bamboo and old corrugated container pulp), which were subjected to a refining action via a PFI mill. The results obtained on softwood pulps, demonstrated that refining and enzymatic treatments, used in a single way caused changes in the surface, but they were not as effective as when the two treatments were used together, especially in causing external fibrillation. The use of the CellA treatment at industrial scale allowed to by-pass the low-consistency refiner. While marginally increased the refining energy applied in the high-consistency refiner, which saved 50 kwh/t. In the case of hardwood pulp, the fiber structure was affected only when enzyme treatment was combined with the PFI refining action; in these conditions a significant density of fibrils was induced. Refining energy requirement decreased by 24%.

Gil (2009) studied the effect of other type of cellulases (Celluclast 1.5L®), produced by *Trichoderma reesei,* and beta-glucanases (Viscozyme L®), produced by *Aspergillus aculeatus*, both enzymes (1-4 IU) were applied during 60 min on *Eucalyptus globulus* BKP. Pulp degradation was evaluated by tensile strength and pulp drainability; these properties were improved up to 34.4% and 80% respectively with 1-2 IU of enzyme at 50ºC and pH 5 at the same level of refining energy (1500 PFI revolutions). The increment of WRV was lower (17.5%). Higher enzyme concentrations and refining levels implied a decrease of pulp drainability. The assays with Viscozyme L® were performed with the same conditions that were used for the

cellulases treatment. However, this treatment did not show a significant influence on pulp properties at 1500 PFI revolutions. When refining increased freeness decreased by 36%, WRV increased by 7.9%, and tensile index improved by 43.4%.

Ko (2010) studied the refining effects on BKP with two cellulases enzymes, one produced by *Paenibacillus campinasensis* and the other one a commercial product named FiberCare®. FiberCare® is an enzymatic formulation in which endoglucanase (carboxyl methyl cellulose) predominates; the enzyme protein content in the product is 6 mg/mL. This study was carried out with three different pulps: unbleached, oxygen bleached and fully bleached. Operational conditions by cellulase were 40ºC, 0-20 IU/g and pH 7, and by FiberCare® were 40ºC, 185.2 IU/mg and pH 6. The retention time and pulp consistency for both enzymes were 60 min and 10% respectively. The cellulase from *Paenibacillus campinasensis* decreased freeness of fully bleached eucalyptus kraft pulp when applied 3350 and 2350 PFI revolutions to reduce freeness from CSF 621 to 400 mL. That corresponds to a 10% and 37% refining energy savings, respectively. On the other hand, the effects of refining on the average fiber sizes of BKP pulps after 24 h of treatment with FiberCare® showed a reduction of fiber and fine lengths and an increase of fiber widths. Fines content (%) was increased most for the oxygen treated pulp and freeness was reduced from around CSF 708 to 315 mL. Endoglucanase treatment increased freeness for both control and PFI refined pulps (Kerekes 1990).

Lecourt (2010) measured the effect of three different cellulases named Cellulase A (4200 ECU mL^{-1}), B (3800 ECU mL^{-1}), and C (3800 ECU mL^{-1}) on bleached softwood chemical pulp (consistency of 4.5%) before refining. To match with industrial conditions temperature was set to 40ºC and treatment time to 30 min. pH was neutral with no adjustment, the total enzyme applied was 200 g/t. Refining was run in two cycles, all pulp processed in the refiner in the first cycle was stored, then it was homogenised, sampled and transferred to the refiner for a second refining cycle. Drainage index and fiber morphology were measured as a function of energy consumption. Cellulase A and B reduced energy consumption by 30% during refining and also improved fiber characteristics and paper properties. In these successful cases of fiber modification, a more intense fibrillation, a higher conformability and flexibility, and a higher bonding potential were observed. However, to obtain these valuable effects, fiber shortening, intrinsic strength degradation of the fibers and decrease in tear strength of the paper have to be assumed. Perhaps, these negative effects could be mitigated by modifying refiner disk geometry.

The study performed by Cadena (2010), using Cel9B and the truncated forms, which contained various combinations of the functional domains of Cel9B GH9–CBD3c, Fn3–CBD3b, and CBD3b from *Paenibacillus barcinonensis,* on total chlorine free bleached (TCF) pulp from *Eucalyptus globulus* (consistency 10%) also evaluates the enzyme effects on refining. The application of endoglucanase Cel9B was found to accelerate the mechanical refining of pulp in a PFI mill and also to decrease its CSF and to increase its WRV. This effect was observed in a interval of temperature between 40-55ºC at pH 5.5 with a retention time of 60 min. The drainage resistance can largely be ascribed to the catalytic domain of the enzyme; in fact, the truncated form GH9-CBD3c, which contains this domain, increased pulp WRV by 5% and drainage resistance by 25%, while reducing fiber length to a smaller extent. CBD3b increased drainage resistance by up to 9% without altering fiber length or the content in fines. This indicates that this treatment increased external and internal fibrillation. These results suggest that the truncated form GH9–CBD3c caused delamination and/or softening of the outer walls of cellulose fibers, to an extent that increased substantially the effect of pulp refining. Thus, it led to an increased tensile strength, burst index, and, even more interesting, an increased tear index relative to whole Cel9B. Therefore, a reduction of the specific refining energy was observed.

Xylanase named Pulpzyme HC® (produced by *Bacillus* species) was used to modify bleached softwood pulp (consistency 5%) before refining by means of PFI mill. The enzyme was applied at pH 7 and 50ºC. The results showed that the enzymatic treatment decreased the charge of the dissolved fraction and the absolute zeta potential of the slurry and increased the fiber surface wettability, improving the refining efficiency. The brightness and bulk of hand sheets increased as a function of enzyme dosage. Tensile index and tear index of hand sheets reached the maximum at the enzyme dosage of 0.2 U/g (Du 2012). This enzyme has also been used by Ribas (2011). They tested the effect of combining ultrasound and xylanase on *Eucalyptus* kraft pulp. Three types of treatments were performed: (i) ultrasound and subsequent xylanase (XA-

1); (ii) xylanase and subsequent ultrasound treatment (XA-2); (iii) xylanase only. In the three cases a pulp at 3% consistency, pH of 7-7.5 and 23ºC; was treated with 5000 U/Kg of enzyme. After the treatments, the pulps were refined in a PFI mill laboratory trying to achieve a value close to 206 mL CSF. The minimum CSF achieved was 142 mL. It was observed that the idea of ultrasound acting as facilitator of the action of enzymes cannot be affirmed, since for most of the properties the effects of XA-1 and XA-2 were statistically equal. It was nevertheless noticed that the junction of ultra-sound and xylanase improved tensile index, specific elastic modulus and tensile energy absorption and decreased tear index of handsheet and that, if ultrasound was applied before xylanase, it also increased opacity.

Bolmstedt (2010) demonstrated that the xylanase enzyme 13-200 nkat/g improved dewatering properties. The enzyme was applied at 50ºC and pH 7.5, during 30 min to a pulp with a consistency of 4-5%. The time to reach the 95% dry solids decreased by up to 15%, which can lead to reduce energy consumption in the dryer section. On the other hand, the freeness of the enzyme treated pulps increased. The dewatering in forming section or in the first press nip was not improved. This is possibly due to a higher flexibility of the enzyme treated fibers, producing higher wet web density, lower porosity and permeability, and consequently higher filtration resistance at flow rates comparable to the paper machine. The effect of the xylanase treatment on paper strength properties was only minor and neither the pulp yield not the fiber strength was compromised. The pulp brightness was improved, which could be beneficial, permitting a reduced use of bleaching chemicals or optical brightening agents.

Bleached eucalyptus and *Pinus radiata* kraft pulps were treated with laccase supplied by Novozyme (NS51003), which was used in combination with various mediators: syringaldehyde (SA) or a new mediation (NM). Laccase solution was added in the pulp suspension during the slushing step, before refining, at a pulp consistency of 4.5%. In order to match with industrial conditions, temperature was fixed at 40℃ and treat ment time at 30 min. pH was neutral without adjustment. On a *Eucalyptus* bleached kraft pulp, laccase associated to SA or to NM affected the refining behaviour. Lower bulk values were measured for the biotreated pulps, fibers were more flexible and more amenable to compaction during the sheet formation. The tensile strength was improved with the new mediator and laccase treatments. Besides, some impact on the fiber intrinsic strength was observed. The laccase associated to NM allowed to enhance breaking length, tear index (10%) and energy savings (30%) (Lecourt 2011).

The use of laccase from *Trametes villosa* and the mediator hydroxybenzotrialoze (HBT) in TCF pulp removes hexenuronic acids (HexA) by 23% and reduces brightness reversion by 8.4%. This treatment was applied at 30ºC, and pH 4, during 30 min. Additional tests conducted to assess the effect of HexA on pulp refining revealed that these oxidizable structures introduced hydrophilicity in the pulp. Removing HexA from TCF pulp alters its refining outcome in terms of drainage resistance and water retention capacity; it also leads to paper with comparable strength-related properties which require no additional refining energy. The fact that the oxidative system has no effect on fiber morphology allows one to ascribe changes in paper strength to electrokinetic properties. Thus, Laccase alters the zeta potential and cationic demand (surface charge) of pulp, thereby reducing its ionic charge and repulsive forces between fibers and fines as a result (Cadena 2010a, 2010b).

Enzymatic effects on secondary fibers

As it is well known, with each recycle, the quality of the raw materials deteriorates because of the undesirable changes in the fibre properties and the higher drainage resistance of recycled pulps. The first affects interfiber bonding and consequently paper strength. The second makes sheet formation more difficult, decreases the paper machine runnability and increases drying energy consumption because the recycled fibers have higher drainage resistance than virgin fibers due to its exposures to repeated pulping and drying process (Rashmi and Nishi 2010).

Modification of secondary fibers by using enzymes has been an interesting topic in the last years. These modifications can result in a substantial increase in pulp freeness with little or no loss in physical properties.

The recycling of paper is an area where the use of enzymes has been implemented, seeing that the recycled fibers' properties can be improved by treatment with cellulases and hemicellulases. In fact, these enzymes modify the interfacial properties of fibers, increasing the affinity for water, which in turn promotes changes in the technical properties of the pulp and paper, such as pulp drainability and paper strength. These enzymes are also used for upgrading secondary fibers'

deinking (Gil 2009, Ibarra 2011).

Valchev and Bikov (2011) studied the effect of FiberCare® D (endoglucanase activity) treatment on the dewatering time and refining degree of Duropak –Trakia-Papir old corrugated container (OCC) pulp (consistency 6-10%) shown that the enzyme charge 0.05 – 0.2 % applied at 60ºC, in a pH range of 4-7, during 60 min, improved pulp dewatering by 20 - 45% and refining degree up to 25%, respectively. In paper production, dryer steam consumption could be reduced by over 4%. The effect of enzyme treatment shows that breaking length slowly increased with enzyme dosage, while tear index and burst index decreased. The obtained results can be interpreted to be due to partial degradation of colloidal substances of secondary fibers. That gel fraction retains water, causing slower dewatering of secondary fibers. A similar study of cellulase action on the pulp dewatering was conducted with deinked pulp. Typical feature of that pulp is the presence of fillers (basic $CaCO_3$), pulp additives and mechanical fibers. A lower enzyme activity was found for this pulp, which makes it possible to add a higher amount of FiberCare® D and still obtain the same dewatering. The breaking length of the fibers increased slowly with the dosage of enzymes, while the tear and burst indexes decreased. Probably at low enzyme dosage, the FiberCare® D action contributed to an improve paper structure independently of fiber destruction processes.

On the other hand, Huang et al (2010) demonstrated that the effect of cellulase-5® (endoglucanase) pre-treatment on refining of the same pulp depended on the type and dosage of enzyme, pre-treatment time and pH value and that influenced greatly the effects on refining. The optimal conditions for treatment evaluated in this study were enzymatic dosage of 0.05%, pH 7.7 and retention time of 47 min. The refining degree, ring pressure index and tensile index of the secondary fiber pretreated with were 44.4%, 18.6% and 25.2% larger than those of the pulp without pretreatment with the cellulases.

Bajpai (2010), commented other case where an enzymatic mix included in the commercial product Fiberzyme LBR® (cellulase/ hemicelullase) had a positive effect on a double-sorted OCC pulp treated (consistency 4%). At 50ºC, enzymatic dose 0.05%, pH interval 5-7 and retention time 180 min, this commercial product decreased the energy consumption by 30%, to reach 429 mL CSF.

One of the important roles of the amylase in pulp pretreatment and refining is to remove fines and impurities from the secondary fibers. The refining of secondary fibers from OCC with commercial amylase can increase their mechanical strength. The addition of 1% of amylase in the refining process increased the ring crush index and tensile index of the refined fibers by 18.3% and 15.6%, respectively (Huang 2010).

Bhardwaj (1996) tested different commercial enzymatic products on a mixed pulp consisting of 60% waste corrugated kraft cuttings and 40% softwood. The main activity of these commercial products is xylanase and the optimal conditions are summarized in table 5. The majority of enzymes obtained a reduction in refining time by about 17-22%, without affect strength properties. With hemicellulase ‘Amano’ 90 the refining time was reduced by about 25% and the drainage was improved by 40%, but the strength properties were slightly affected (3). Poorna and Prema (2007) demonstrated that the effect of crude enzyme on waste paper utilization reached a significant change on the surfaces; the surface of untreated pulp appeared smoother than that of xylanase treated fibers. Fibers of treated pulp underwent a peeling process giving rise to flakes and filaments of materials detached from fiber surfaces, due to xylan hydrolysis. Enzyme treatment increased the fiber swelling, which facilitated refining, which in turn resulted in better physical properties. Enzyme addition prior to refining can improve strength properties at a fixed refining level.

BIOFILM

Biofilms in the paper industry can occur at solid–liquid (deep down in chests and in pipes), solid–air (on the machine parts where no liquid water are flowing), liquid-liquid (agglomeration in the water phase) and liquid–air (on the aerobic interface where water levels are continuously changing) interfaces. A biofilm is a structured community of mixed species enclosed in a self-produced extracellular polymeric matrix formed by exopolysaccharides (EPSs) and adhered on an inert or living surface (Lopez 2010; Torres 2011; O´Toole 2000; Watnick 2000). EPSs are composed of a wide variety of species including polysaccharides, proteins, nucleic acid, uronic acid and humic substances. This configuration explains that bacteria in biofilm show greater resistance to biocides compared to their planktonic forms. Established biofilms can tolerate

antimicrobial agents at concentrations of 10-100 times higher than those needed to kill genetically equivalent planktonic bacteria, making biofilms extremely difficult to eradicate (Burmolle 2006, Jefferson 2004, Stoodley 2002).

EPSs also serve many other functions such as: providing a structural integrity, bacterial protection and intercellular communication, formation and maintenance of the micro colony and also enables the bacteria to capture nutrients. EPSs act as a barrier against hostile environments. EPSs have a complex architectural structure (Flemming 2002) containing channels which allow the inflow of water, oxygen and nutrients and outflow of by products and enhances bacterial resistance to antimicrobial agents, the production of EPSs is influenced by internal and external factors including: Quorum sensing (cell to cell communication), surface topography, hydrodynamic shear forces; fluid velocity and nutrient availability. The difference in the quantity of biofilm EPSs is a result of the growing conditions of the biofilms (Molobele 2010).

Since natural biofilms are composed of mixed species and several different complex EPSs structures it is evident that enzymes mixtures able to degrade these complex structures need to have a wide variety of highly specific enzymes and therefore, are not always commercially available. Due to this, most enzymes acting on EPSs have to be isolated from 3 major sources: endogenously from EPSs synthesising microorganism, exogenously from a wide range of other prokaryotic and eukaryotic microorganism or from bacteriophage particles or phage induced bacterial lysates. Enzymes are usually highly specific (Simoes 2010, Torres 2011, Verhoef 2005).

The major enzymatic antifouling mechanisms include: cell lysis by degradation of components of the cell membrane, degradation of compounds anchoring cells to the surface: (i) of adhesives produced during settlement and anchorage and (ii) of the extracellular matrix secreted by proliferating adhered organisms (Oulahal 2007, Van Houdt and Michelis 2005), disruption of intercellular communication: quorum sensing, i.e., bacterial cell–cell communication (Kristensen 2008) and degradation of environmental substances (i) that are fundamental for the survival of the fouling organisms or (ii) generating antifouling compounds (Cordeiro 2011).

There are two main alternatives to develop effective enzyme products for application in the paper industry. The first one involves directly identifying the polysaccharides present in the slime and looking for specific enzymes able to degrade them (Verhoef 2003). An example of this case is the levan, which is a β-2,6-linked polymer of fructose that forms a slime film. This compound is secreted by several species of *Bacillus* and *Pseudomonas* bacteria that can grow in paper machines, especially in those manufacturing fine paper, where the level of inhibiting compounds is low. The enzyme levan hydrolase can hydrolyze this polymer to low-molecular-weigth polymers that are water soluble, thereby cleaning the slime out of the system.

Other case where was applied is a family of products called Darazyme, developed by Grace Dearborn´s group, this work was based in the preliminary identification of slime components and the subsequent application of specific enzymes or combination of enzymes depending on the polysaccharides found (Bajpai 1999).

Verhoef (2005) showed the presence of the EPSs colanic acid in several different paper mills from Spain and Finland. Colanic acid is commonly known to be produced by several members of the *Enterobacteriaceae* family. Later, described the characterization and partial purification of a novel β(1,4)-fucanosyl hydrolase that causes a 100% conversion of colanic acid to its corresponding hexasaccharide repeating unit. Furthermore, enzymatic research revealed new insights in the O-acetylation pattern of colanic acid. The fucoside hydrolase purified was an endo acting, hydrolytic enzyme cleaving colanic acid between the two adjacent fucopyranosyl residues present in the backbone of colanic acid resulting in the formation of single repeating units with varying degrees of O-acetylation. Kinetic studies and substrate modifications revealed that the affinity of the enzyme for colanic acid is based upon electrostatic and hydrophobic interactions and that removal of the O-acetyl moieties results in an increase in catalytic activity (Verhoef 2003).

The second alternative is indirectly testing to identify enzymes that show activity on the biofilm by assessing their effects on slime development. The specificity in the way enzymes interact with the biofilm makes this a complex technique, being rather difficult to identify enzymes that are effective against all the different types of slimes. Formulations containing several types of enzymes seem to be fundamental when it comes to applying this slime control approach

successfully (Simoes 2010).

To gain more understanding on the chemistry of biofilm attachment Kolari (2003) investigated the sensitivity of *Deinococcus geothermalis* E50051 biofilms (specie isolated from paper mill) towards enzymes that hydrolyze macromolecules expected to represent components of its biofilm matrix. The treatment with pronase (a broad-spectrum protease from *Streptomyces griseus*) for 2.5 h detached *D. geothermalis* biofilms from the surfaces of glass and polystyrene, and polypropylene. When the buffer solutions were plated on TSA agar after this 2.5 h treatment, a higher number of cells grew out of the enzyme-buffer solution than of the buffer with no enzyme (the negative reference). Light microscopy showed that in the enzyme-buffer solution there were single cells rather than cell clusters. These findings seem to point out that protease treatment released intact living cells from the biofilm matrix, and that proteins are involved in the cell-to-cell attachment of *D. geothermalis* in biofilms. Enzymes such as amylases and proteases have been found useful in enzymatic boil out of slime encrusted equipment. One approach to the removal of slime from equipment is to produce sufficient slime from organisms isolated from the source, set up enrichments, and select organisms that will grow on it. Organisms were found that could degrade these polymers depending on the polysaccharide and organism used to make it. This approach may provide a source of new enzymes to be used for cleaning such deposits (Kenealy and Jeffreis 2003).

Torres (2011) tested 17 commercial enzymatic products on biofilm formed by the flora present in the process water obtained in the sheet forming zone of the wire section from a 100% recycling paper mill using mixed recovered paper as raw material and producing paper for board. The results showed that Pectinex Smash® and its fraction Novoshape®, were the best formulations in the prevention of biofilm formation (having in common that they are both principally composed by the pectin methylestearase). Pectinex Smash® has a mixture of pectynolitic activities and Novoshape ® is an enzymatic solution of a microbial pectin methylesterase (PME, E.C.3.1.1.11). The gene encoding of the esterase enzyme is derived from fungus *Aspergillus aculeatus,* and is transferred into a strain of the food-grade organism *Aspergillus oryzae* for commercial production. The NovoShape preparation has a declared activity of 10 PEU/mL and an optimal temperature of ~50 C (data provided by the manufacturer). This enzyme belongs to the carbohydrate esterase family, catalyses the hydrolysis of methyl ester groups and has high specificity for pectin substrates, property widely used in food industry and in plant science.

These studies open the way to devise a method of removing biofilms from surfaces submerged in water using an effective enzyme that could be combined with the use of a biocide (Stodley 2002). The biodegradability and low toxicity of enzymes make them attractive biofilm control agents. Various approaches are being used to increase the stability of enzymes, including enzyme modification, enzyme immobilization, protein engineering and medium engineering. Although these conventional methods have been frequently employed to improve the stability of enzymes, various new techniques, such as self-immobilization of enzymes, the immobilization of enzymes on nano-scale structures and the production of single-enzyme nanoparticles, are gaining a great deal of attention at present (Richards and Cloete 2010).

Conclusions

Enzymes are catalysts of biological origin that meet many requirements to improve different processes in multiple industrial sectors, among them, the pulp and paper industry. Their use has increased in the last few years thanks to the progresses made in genetic engineering (microorganisms modification) and biotechnology. The use of enzymes in the paper industry presents a number of advantages both economic and of technological nature.

Enzymes as refining / modification agents: Before the sheet formation stage, the fibers need to be refined to a sufficient degree to obtain an optimal fibrillation. Enzymes have been proven to have a refining effect, this being dependent on the particular enzyme type, substrate, and application conditions. If treatment applied is limited, the enzymes remove only elements having a great affinity for water but contribute little to the interfiber binding potential. By selectively removing these surface components, pulp-water interactions are reduced and drainage increases without noticeable changes in the final mechanical strength properties of the pulp. If the treatment is extended, however, fibrillation becomes pronounced and drainage decreases. Moreover the treatment with enzymes before refining reduces the specific energy requirements of the process and the generation of fines; the treatment after refining improves dranaibility of

pulp resulting in an enhanced runnability of the paper machine and a decrease in the consumption of steam in the dryer section of the machine.

Enzymes as biofilm control agents: the application of enzymes to control biofilm in the paper industry is not widely known. The research efforts on this topic have been focussed on characterising the different types of extracellular polymeric substances (EPSs) produced by microbial communities to devise strategies for the removal of biofilms. The use of enzymes to remove bacterial biofilm is still limited, partly due to the low prices of the traditional chemicals used in the industry for with purpose. Other factors limiting the usage of enzymes as biofilm control agents in the paper industry are the lack of reliable techniques for the quantitative evaluation of the enzymatic treatments effects and a relatively restricted commercial accessibility to certain enzyme formulations. It is known that monocomponent enzymes can be used for biofilm removal. However, the heterogeneity of the biofilm matrix limits the potential of specific enzymes, being therefore necessary to apply a wide range of enzyme activities to achieve an acceptable degree of removal in complex biofilms.

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